A COMPARISON OF LEAF SUCROSE ACCUMULATION AND REGULATION BETWEEN PLANTS OF *C. PLANTAGINEUM* DRIED IN THE LIGHT AND DARK

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ABSTRACT

The resurrection plant *C. plantagineum* is able to survive almost complete water loss in their vegetative tissues and can then rehydrate rapidly on rewatering. The ability to recover completely from dehydration has been attributed in part to the ability of the plant to accumulate large quantities of sucrose. The accumulation of sucrose stabilises membranes, protects proteins and contributes to cellular osmoregulation during water stress. However, it has been observed in *C. wilmsii* by research group of Farrant (unpublished) that sucrose accumulations differ significantly when dehydration is carried out in the light and the dark. Farrant (unpublished) observed a significant increase in sucrose accumulation when dehydration was carried in light while dehydration in the dark resulted in very low amount of sucrose. Based on these findings, using the plant *C. plantagineum*, metabolic factors, which could contribute to differences, observed in *C. wilmsii* for sucrose accumulation in both light and dark was examined in this project. To achieve this, three enzymes in relation to sucrose accumulation from leaves dried in both light and dark treatments were examined, namely: hexokinase, acid invertase and aldolase. The amount of glucose, fructose and sucrose during the dehydration process in both light and dark treatments were also examined. Results obtained show a significant decreases in sucrose (9.8-fold decrease) when tissues of *C. plantagineum* where dried in the dark as opposed to the light. Furthermore, the amount of hexose sugars was also significantly lower in dark dehydrated tissues. Moreover, the enzyme data for dark dehydrated samples showed that both aldolase and hexokinase activity levels were significantly low. In contrast, enzyme data for the light dehydrated tissues showed that aldolase and hexokinase activities were maintained, although a higher level of aldolase activity was recorded, until approximately 10% RWC where a drop in activity was recorded for both enzyme. However, acid invertase activity was comparable for both light and dark dehydrated tissues. Thus it seems that the down regulation of aldolase during dehydration in the dark might be responsible for the low sucrose content obtained in the dark for *C. plantagineum*.

Introduction

In nature, plants have evolved different ways of coping with their environment. One of the major threats for survival of all vegetative plants is the limitation of water. Water is the absolute constituent for life. It is needed for most biochemical and metabolic reactions. Consequently, plants have evolved different ways of dealing with water deficit stress. Some are drought evaders for example ephemerals, some are avoiders like the succulents, while others tolerate the stress like resurrection plants. Resurrection plants are also commonly known as desiccation tolerant plants and an example is *Craterostigma plantagineum.*

There are two different types of vegetative desiccation-tolerant plants: -

1) Modified desiccation-tolerant plants that can only survive desiccation if drying rates are slow (Oliver *et al.*, 1998).
2) Fully desiccation-tolerant one that can survive the loss of free protoplasmic water at any rate (Oliver *et al.*, 1998).

Modified desiccation-tolerant plants include members of plants from the more complex groups of ferns to angiosperms with only a few members in the less complex bryophytes and algae being classified as modified desiccation-tolerant plants (Oliver *et al.*, 1998). These plants use an array of morphological and physiological mechanisms to retard water loss to the extent required to establish tolerance.

Well-known and relatively well-studied member of the modified desiccation-tolerant plants are the *Craterostigma spp* and *Xerophyta spp.* Resurrection plants can tolerate almost complete water loss in their vegetative tissues making them quite remarkable in this respect. For example, in *Craterostigma spp*, fully mature leaves can lose up to 95% of their relative water content and enter a dormant state and upon rewatering, the leaves are rehydrated and fully photosynthetically active within 24 hours (Sherwin & Farrant, 1996, Norwood *et al.*, 2000). Tissue damage during drying and rehydrating processes seem to be minimal to non-existent (Scott, 2000). In this way, the plant is preserved until water becomes available; at this point, it is ready to take immediate advantage of the prevailing

conditions. Consequently, resurrection plants have a greater competitive advantage over other species for certain ecological niches.

As stated earlier, resurrection plants use an array of morphological and physiological mechanisms to prevent damage associated with water deficit stress. Moreover, during the stress period, the presence of light can be extremely damaging to the plant. Presence of light will drive photosynthesis which very often results in the formation of oxygen free-radicals formation namely the so called reactive oxygen species (ROS); due to the inability to transfer excitation electrons from chlorophyll to photosystem II and also from reactions within photosystems causing formation of superoxide and mono-oxygen (Sherwin & Farrant, 1998). The, formation of ROS can result in severe subcellular damage to the subcellular millieu. *Craterostigma spp* avoid this problem, in part, by masking its chloroplasts from direct light during drying. Therefore, during drying the leaves curl inwards and only the abáxial surface of the outer rosette leaves become exposed to light. Also, during this process, the plants produce a lot of "sun-screen" pigments, for example, anthocyanins, which are mostly found in the abaxial surface (Farrant, 2000). This pigment, purple in colour, reflects light, thus protecting the photosynthetic apparatus of the plant. It must be noted that beside the role of reflecting light, anthocyanin is also considered to be an antioxidant, therefore, accumulation of this pigment not only protect chlorophyll from light-driven photosynthesis but also help minimise damaged associated with free-radical formation (Farrant, 2000). Furthermore, Farrant (2000) also shows that photosynthesis declines at higher relative water content (RWC) during dehydration. Thus on the onset of water-deficit stress, photosynthetic activity start declining when the RWC is 80% until around 60% where it stop completely (Farrant, 2000). However, during this period respiration is still occurring and only stops when the RWC drops to 10%. It must be noted that respiration is equally damaging to the plants as it also results in ROS formation. Consequently, it has been proposed by Farrant (2000), that this process allows energy to be produced, which is needed to put down the different protective mechanisms, but that anti-oxidants are also needed to neutralise the ROS.

Why is sugar important during stress?

All resurrection plants accumulate large amount of compatible solutes, among them sugars, on drying. This factor seems to be important in the acquisition of desiccation tolerance of the protoplasm (Wolfram *et al.*, 1998). Most investigations carried out so far, have shown a decrease in the concentration of starch and subsequent increase in sucrose accumulation on dehydration (Norwood *et al.*, 2000; Scott, 2000 & Whittaker *et al.*, 2001). Moreover, it has also been shown that most resurrection plants deplete monosaccharride sugars,which is thought to be a protective mechanism against free-radicals formation (Farrant & Sherwin, 1998). Thus, it seems most likely, that accumulation of soluble carbohydrates during drying plays an important role in acquiring desiccation-tolerance in resurrection plants.

Two hypotheses have been put forward to account for the accumulated sugars: -

1) In the dry state sugars could act by maintaining hydrogen bonding within and between macromolecules thus stabilising membranes and proteins of the plant (Scott, 2000) – The Water Replacement Theory (Crowe & Crowe, 1980).

2) Sugars could put a stasis on the cell contents and stabilize internal cell structure (Scott, 2000) – The Glass Formation Theory (Vertucci & Farrant, 1995). Only on addition of water will this glassy state melts, and metabolism can resume.

Recent evidence has shown that a combination of these two processes is important for the maintenance of cell integrity during dehydration (Crowe *et al.*, 1998). Moreover, it has also been suggested that sugars alter the physical properties of dry membranes so that they resemble those of fully hydrated biomolecules (Ingram & Bartels, 1996).
Thus in summary, the accumulation of sugars stabilizes membranes, protect proteins and contribute to cellular osmoregulation during stress.

So far its been shown that all resurrection plants accumulate large amounts of sucrose during stress. Amazingly, it was found that *Craterostigma spp* namely *C. plantagineum* & *C. wilmsii* not only accumulate sucrose but concomitant with this accumulation is the mobilisation of 2-octulose reserves in the leaves (Norwood *et al.*, 1999). 2-octulose is an

8-carbon sugar, produced during photosynthesis. Normally, in *Craterostigma spp*, 2-octulose is accumulated in leaves over the light period and mobilized at night. Thus this 8-carbon carbohydrate molecule acts as a temporary storage in leaves and upon drying, these accumulated molecules are rapidly converted to sucrose via the gluconeogenesis pathway by the enzyme aldolase, glucose-2-phosphate dehydrogenase and fructose-1,6 bisphosphate, which are key enzyme in this process (Norwood *et al.*, 2000). It has been proposed by Scott (2000) that the conversion of 2-octulose accounts for up to 80% of carbohydrate accumulation in leaves of *Craterostigma*. Hence, *Craterostigma spp* can accumulate sucrose very rapidly from carbohydrate sources already present in the leaf rather than relying upon photosynthetate. Moreover, it has also been proposed that during dehydration in *Craterostigma spp,* the older leaves die and carbohydrates found in these leaves are translocated to the mature leaves and thus contribute to the accumulation of sucrose. Additionally, carbohydrates found in senescing leaves and root tissues, also contribute to sucrose accumulation.

Thus during stress, the principle sugar being translocated is sucrose, which enters the leaves as a result of hydrolysis by the enzyme invertase (cell wall invertase and neutral invertase). The glucose and fructose resulting from the hydrolysis of sucrose would then be phosphorylated by hexokinase and fructokinase, and sucrose synthesized from the hexose phosphates. Another source of carbon to sucrose accumulation in leaf tissue is triose phosphates, which are produced directly by photosynthesis and then translocated to the cytoplasm. Furthermore, it seems that 2-octulose is a major source of carbon to sucrose during the onset of water-deficit stress in *Craterostigma* spp.

Aim

The aim of this project is to compare leaf sucrose accumulation and regulation between plants of *Craterostigma plantagineum* dried in the light and dark. Unpublished data by the research group of Farrant has shown that in *C. wilmsii* tissues dried in the light have higher sucrose content (445 ± 44.9 μmol. gDM^{-1}) than in the dark where only 176 ± 38.0 μmol. gDM^{-1} was observed. Furthermore, Farrant (unpublished) also observed that dehydration in the dark resulted in an increase in hexose sugars (140.3 μmol. gDM^{-1}). In contrast, the total amount of hexose sugars for light dehydrated tissues were only 92.8 μmol. gDM^{-1}. The fact

that total sucrose content for the light far exceeds the total amount sucrose formed in the dark, raise the question of what could affect sucrose accumulation in the dark. Moreover, the fact that total hexose sugars in the dark are far lower than the amount of sucrose formed in the light dehydrated tissues; raise the possibility that there might be other carbon sources contributing to the increase in sucrose observed for the light. Furthermore, plants dried in the dark do not survive desiccation (Farrant, unpublished).

Thus, a further aim of this project is to determine the metabolic factors that might contribute to the difference in sucrose accumulation observed between light and dark treatment in *C. wilmsii* (Farrant, unpublished). These will include, measuring the activity of three enzymes in relation to sucrose accumulation from leaves dried in both light and dark treatment, namely: - 1) Hexokinase

2) Invertase

3) Aldolase

The amount of sucrose, glucose and fructose produced during dehydration in both treatments will also be examined. It must be noted that for this project *C. plantagineum* was used and is assumed that both species behave similarly.

Hexokinase

This enzyme catalyses the phosphorylation of hexose sugars namely glucose and fructose, which are produced during catabolism of sucrose (Theodore *et al.*, 1998). It also regulates carbohydrate entry into the glycolysis pathway and is known to be a sensor for sugar responsive gene expression.

In *Craterostigma spp*, glucose in the leaf material may be derived from the breakdown of imported sucrose from the root. It may also be derived from minimal starch breakdown in the leaves. However, glucose is not completely incorporated into sucrose in leaves dried down in the dark (Table 1). Is this because leaf hexokinase is not upregulated during dehydration in the dark? How do enzyme activity profiles compare in the leaves of plants dried in the light and dark?

Acid Invertase

This enzyme hydrolyses sucrose stored in the vacuole and results in the production of glucose and fructose. If acid invertase activity is higher in the dark, this may account for the low level of sucrose obtained for the dark treatment in table 1. In combination with a lower level of hexokinase activity, glucose levels would also be higher. The relative relationship between acid invertase (responsible for hydrolysing sucrose) and hexokinase activity (responsible for phosphorylating glucose and fructose for sucrose synthesis) is informative in ascertaining whether sucrose is more likely to be broken down or stored.

Aldolase

Even if all the hexose (glucose and fructose) sugars were incorporated into sucrose in the dark, the overall sucrose content in the dark would still be lower than that shown for light dried leaves (table 1). This result shows that there must be other carbon sources beside glucose contributing to sucrose accumulation. In *Craterostigma spp*, 2-octulose is broken down to triose phosphate, which is then channelled via gluconeogenisis into the hexose phosphate pool and sucrose synthesised. Therefore, aldolase, glucose-3-phosphate dehydrogenase and fructose-1-6-bisphosphatase are potential key enzymes controlling the flux of carbon during the conversion of 2-octulose to sucrose. It is possible that these enzymes are down regulated in the dark and therefore are unable to convert octulose to sucrose.

MATERIALS AND METHODS

Plant description

Craterostigma plantagineum (Scrophulariaceae) is a small herbaceous plant, growing from a rhizome with a basal rosette of spreading, broad – elliptic and often serrate leaves (Figure 1.1) (Blundell. 1992). This plant bears blue to violet flowers, which are borne at the end of single stems and resemble violets (Blundell. 1992). Normally grows on shallow soils in open sunny places and dry grassland. During drought conditions the plant can dry almost completely and in the process, they curled their leaves forming a rosette like structure with anthocyanin deposition on the abaxial surface (Figure 1.2). The first drops of rain caused rapid recovery.

Figure 1.1: Fully hydrated *C. plantagineum*

Figure 1.2: Dehydrated with anthocyanin on abaxial surfaces

Plant Materials

The resurrection plant, *C. plantagineum*, was collected from Borakalalo National parks. Two weeks prior to experimentation, plants – 4 pots, were transferred to a plant growth room with a light intensity of 450 μmol m^{-2} s^{-2}, relative humidity (RH) between 50 – 70 %, temperature of 25 °C and a 14H light cycle starting at 6 am till 8 p.m.

Experimental procedures

The following experiments were repeated twice, using four trays each containing approximately 10 plants. Half of the trays were dried in complete darkness (after the soil was dried) under the same temperature and RH as described above while the other half was left under light conditions as previous described.The drying process was initiated by withholding water and allowing the plants to dry naturally (Sherwin & Farrant, 1996). During this process, leaves were harvested at regular intervals (both light and dark treatments) ~~one~~ over a period of two weeks.

Relative water content (RWC) was determined as follows: RWC = water content / water content at full turgor and was expressed as percentage (Farrant, 2000). The water content was determined gravimetrically by oven drying at 104 °C for 24 h – Water content = (Fresh mass-dry mass) / dry mass. Following dehydration, plants were well watered and allowed to rehydrate. In the light? RH?

Carbohydrate measurements

For each total sugar determination, three ~~unpotted~~ unpooled leaf samples were used. To measure glucose, fructose and sucrose (both light and dark treated leave samples), tissues were harvested and immediately frozen in liquid nitrogen and ground to a fine powder. Alcoholic NaOH extraction buffer (100 mM NaOH in 50 % v/v EtOH) was added in a ratio of 1: 80 (dehydrated samples) and 1: 20 (hydrated samples). After incubation on ice for 10 minutes, the tissue extracts were neutralised (pH 7.0 – pH 8.0), by the addition of a buffer containing 100 mM Hepes and 1 M acetic acid. The samples were then centrifuged

(Sorvall Superspeed RC 2 – B) for 15 minutes at 12000 rates per minutes (rpm). The supernatants were removed and the pellet fractions were centrifuged (Sorvall Superspeed RC 2 – B), a second time. The supernatants obtained from each centrifuged were then combined.

The soluble sugars were measured enzymatically. Sucrose, glucose and fructose were determined using the Boehringer Mannheim sugar food analysis Kit (Bergmeyer & Bernt, 1974).

Enzyme Measurements

For each enzymes measured, three unspotted leaf samples were extracted for both light and dark treatments.

Extraction Procedures

Tissues harvested were ground to a fine powder with liquid nitrogen in the presence of equivalent mass of insoluble polyvinylpolypyrrolidone (PVP). To the ground tissues, ice-cold extraction buffer containing 50 mM KH_2PO_4 (pH 7.5), 4 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and 5 mM dithiothreitol (DTT) were added. The ratio of tissue to extraction buffer was 1:20/30. The extracts were then centrifuged (Sorvall Superspeed RC 2 – B) for 20 minutes at 10 000 rpm. To remove metabolites and phenolic compounds found in the extracts, the supernatants were desalted using 2.5ml Sepahdex G – 25 columns (particle size 50 – 150 μm), equilibrated with extraction buffer and centrifuged (Sorvall Superspeed RC 2 – B) for 3 minutes at 200 rpm. The desalted extracts were then used for 3 enzymes analysis namely: I) Hexokinase

II) Acid invertase

III) Aldolase

Hexokinase measurement

Hexokinase activity was measured using a spectrophotometer (Beckman DU 650) at a wavelength of 340 nm in a 1.0 ml volume. The standard reaction is made up of 100 mM KH_2PO_4 (pH 7.5), 2mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 0.4 nicotiamide adenine dinucleotide (NAD), 1 mM adenine triphosphate (ATP), 1.0 IU coupling enzyme (12 μl glucose-6-phosphate dehydrogenase (GLC-6-P Δ) and 6 μl phosphoglucose Isomerase (PGI)), 200 μl desalted extract and 5 mM glucose. The blank contained all the chemicals described above except glucose. The rate of reaction of NAD to NADH was calculated and data was expressed as μmol/min/gDm.

Acid Invertase Measurement

Two replicates of the desalted extracts were incubated at 37°C for 90 and 180 minutes respectively. This allows invertase the enzyme to break down sucrose to glucose and fructose respectively. After the incubation period, the extracts were neutralised with 4 M imadozol (pH 7.6) and placed on a heating block set at 100°C for 30 minutes. This step destroyed all protein present. Glucose produced was determined enzymatically as a measure of invertase activity.

Invertase activity was measured spectrophotometrically (Beckman DU 650) in a 1.0 mL volume. The standard reaction contained 2 mM $MgCl_2$, 1 mM EDTA, 0.4 mM NAD, 1 mM ATP, 40 µl sucrose, 80 µl McIlvanes buffer (pH 5.0) and 40 µl samples. Enzyme activity was calculated from an increase in absorbance as NAD is converted to NADH.

Aldolase Measurement

Aldolase activity was measured spectrophotometrically (Beckman DU 650) at 340 nm in 1.0 volume. The standard reaction consisted of 50 mM Hepes, 1 mM EDTA, 0.1 mM nicotiamide adenine dinucleotide reduced (NADH), 10 IU TPI, 1 IU glycerine-3-phosphate dehydrogenase (G3P Δ), 200 µl desalted extract and 4 mM fructose-1,6-bisphosphate (Fru-1,6-P_2). Enzyme activity was calculated from the rate of oxidation of NADH to NAD.

RESULTS

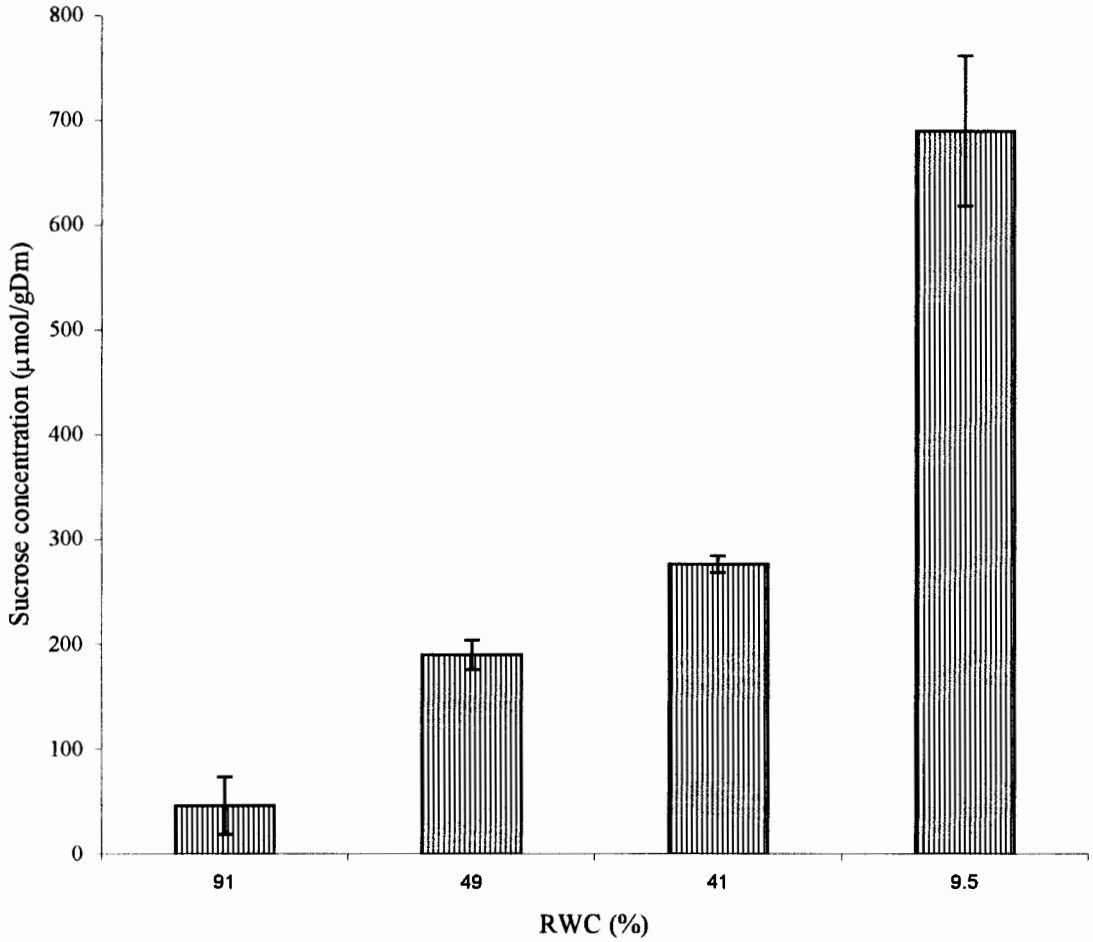

Figure 2: Sucrose levels in C. plantagineum as tissues are dehydrated in the light. The results are the mean ± STD from three replicates of the same plants.

There is a significant increase in sucrose accumulation (Fig.2) as tissues are dehydrated in the light, from 91% to 9.5% RWC. The results show a 15-fold increase in sucrose accumulation. The same trend was also observed by research group of Farrant (unpublished) for *C. wilmsii* although in *C. wilmsii* the amount of sucrose in dry leaves was much less, only 441 ± 44.9 µmol/gDm.

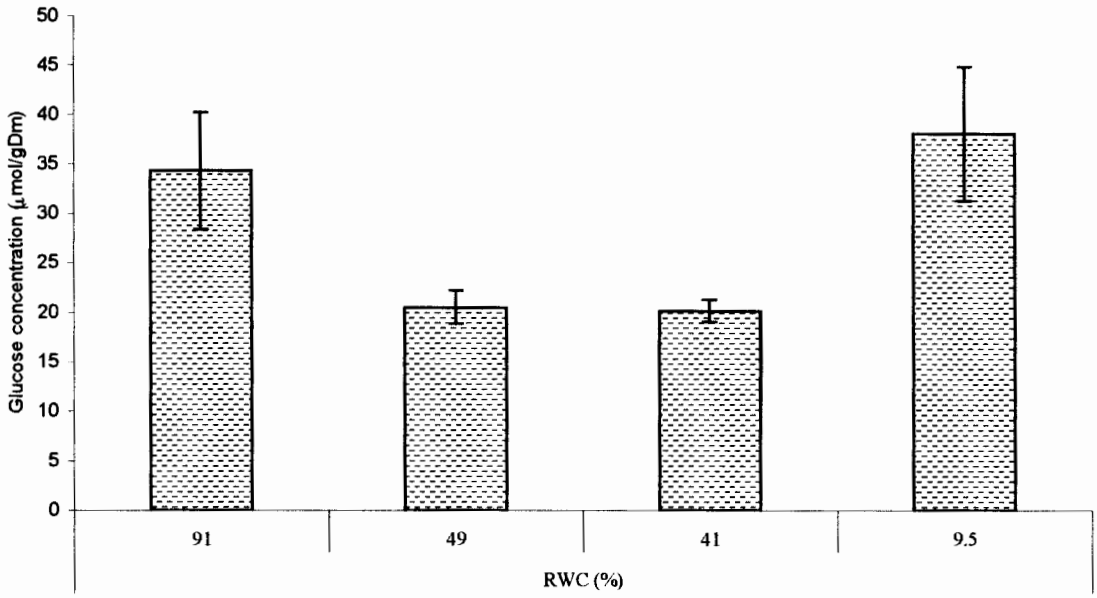

Figure 3a: Trends in glucose levels, as tissues of C. plantagineum are dehydrated in the light. Results are the mean ± STD from three replicates of the same plants.

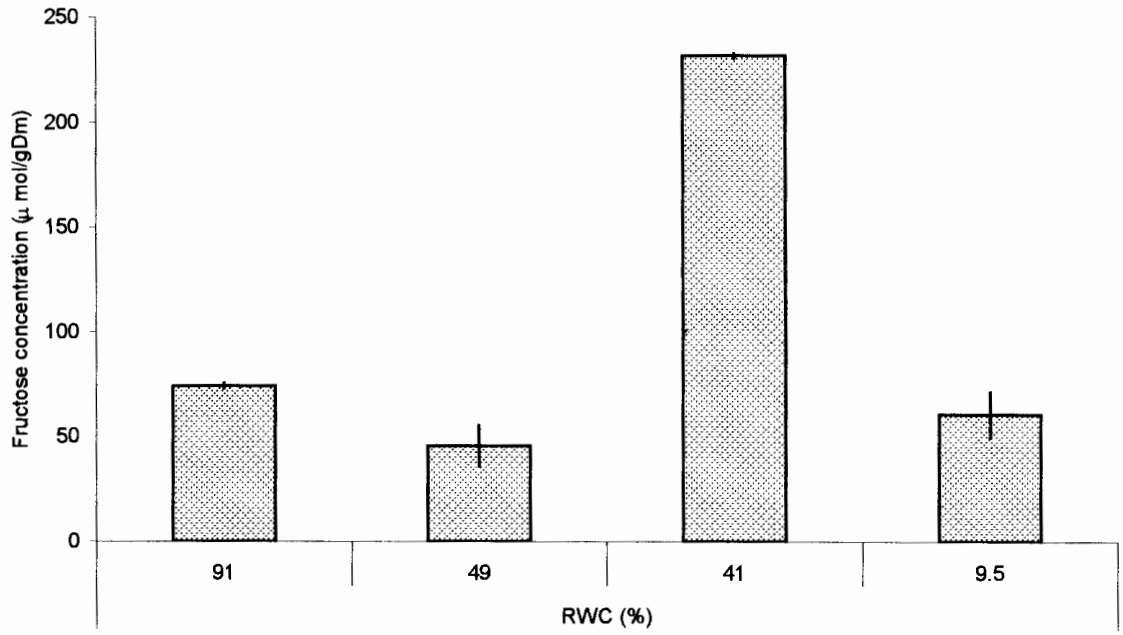

Figure 3b: Trends in fructose levels, as tissues of C. plantagineum are dehydrated in the light. Results are the mean ± STD from three replicates of the same plants.

Figure 3a & 3b show that the amount of hexose sugars remain more or less constant during the process of dehydration except for at approximately 40% RWC, where fructose increases to a maximum of 231.53 ± 1.25 µmol/gDm was obtained. Glucose levels decline somewhat during drying below 90%, but at 9.5% RWC (38.16 ± 6.79 µmol/gDm) levels were comparable with the levels in control tissue (34.31 ± 5.93 µmol/gDm).

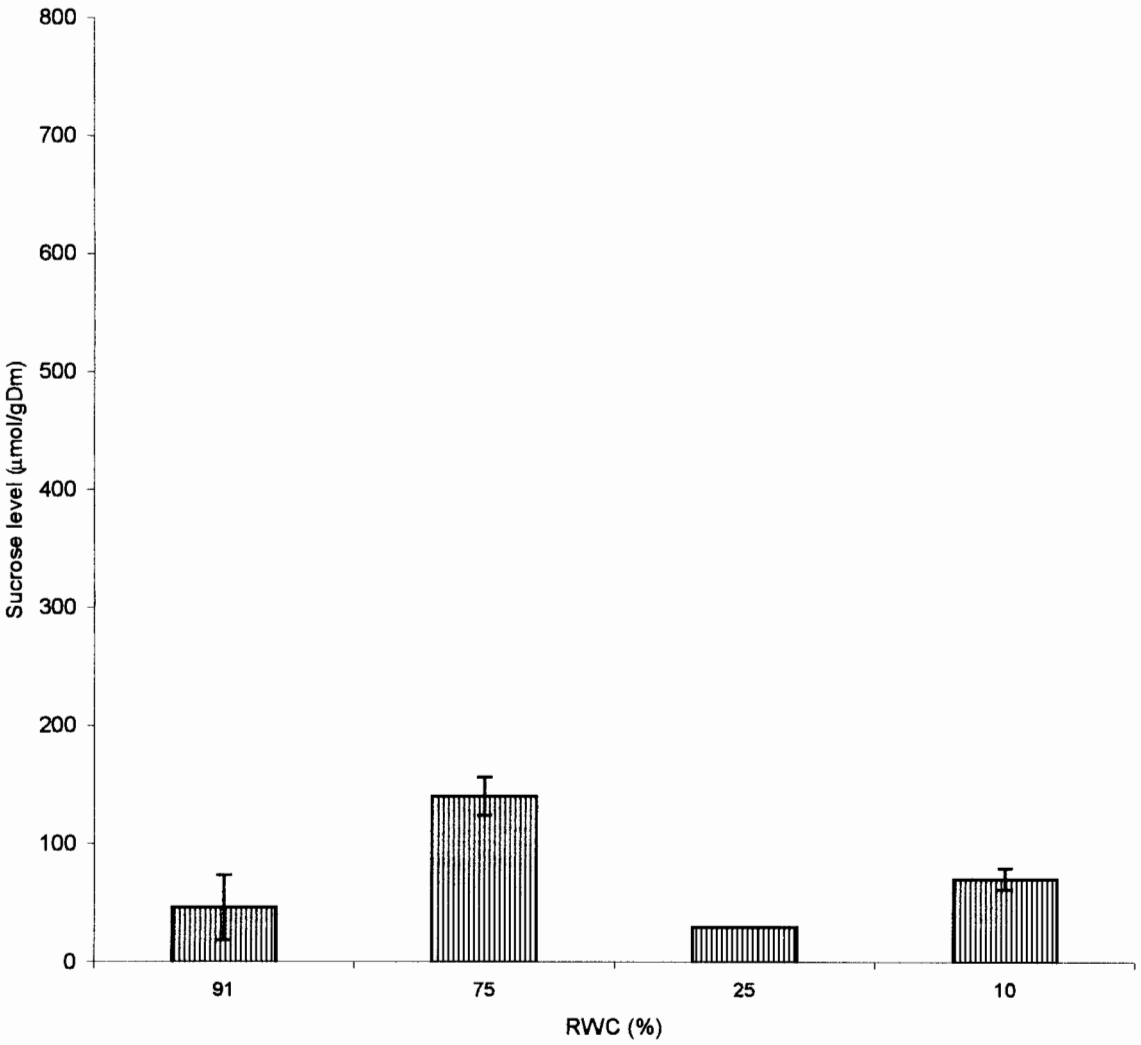

Figure 4: Effect on sucrose accumulation as tissues of C. plantagineum were dehydrated in the dark. Results are the mean ± STD from three replicates of the same plants except for 25% where only one replicate was plotted.

In the dark, sucrose accumulated only slightly during at the early stages of dehydration followed by a decrease thereafter (Fig,4). A maximum of 140.34 ± 15.96 μmol/gDm was obtained at 75% RWC. The value obtained for the fully dehydrated plant material (10% RWC) was comparable with the hydrated tissue.

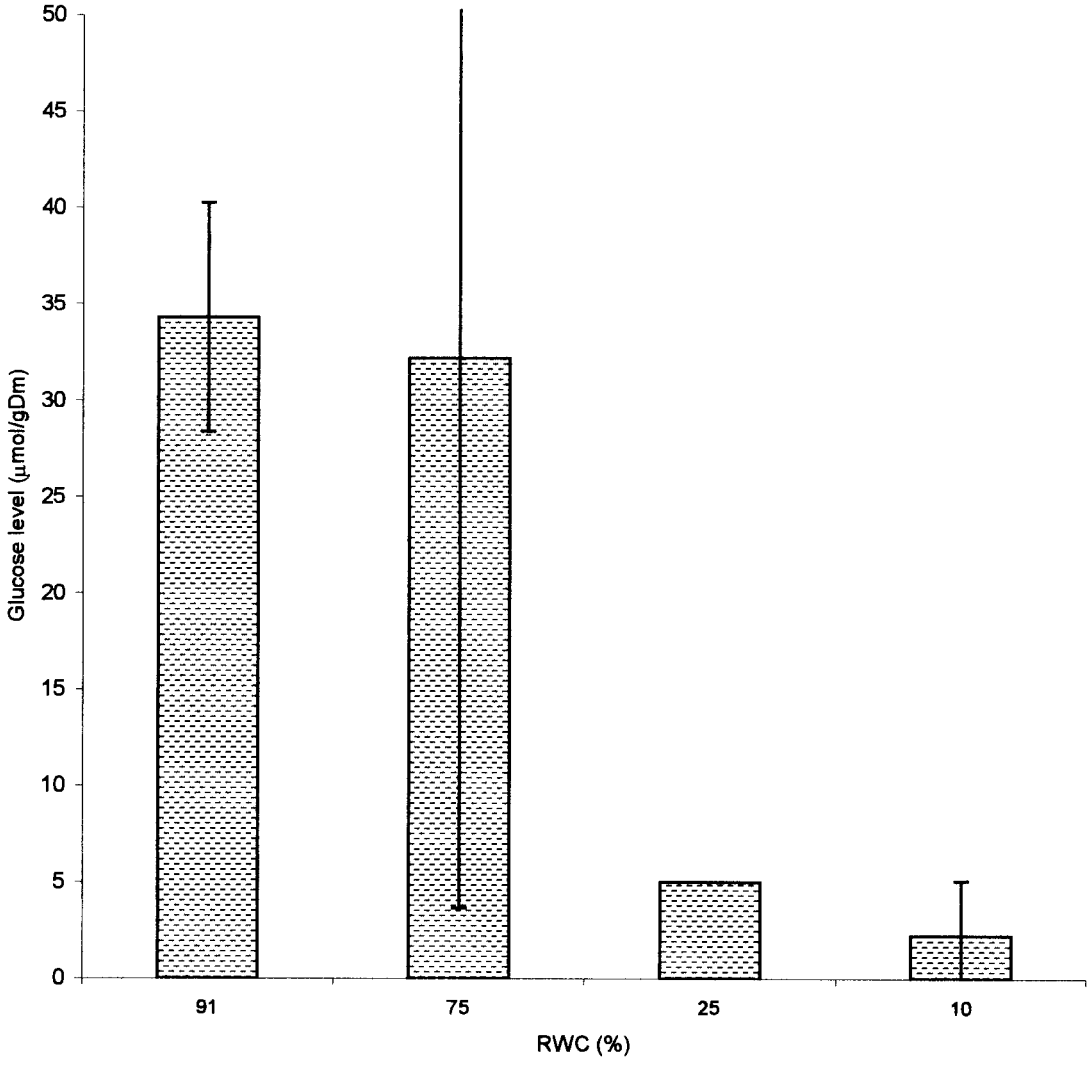

Figure 5a: Trends in glucose levels, as tissues of C. plantagineum are dehydrated in the dark. Results are the mean ± STD from three replicates of the same plants except for 25% RWC where only one replicate was plotted.

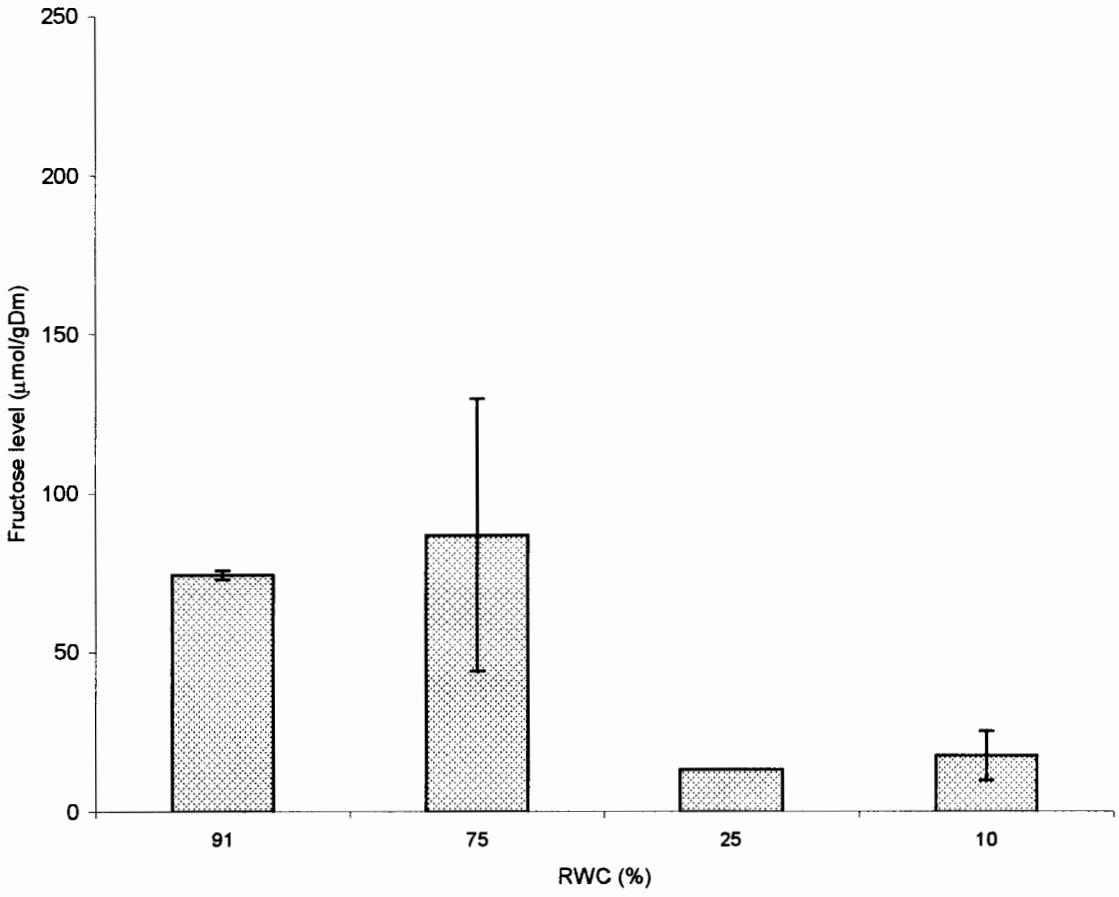

Figure 5b: Trends in fructose levels, as tissues of C. plantagineum are dehydrated in the dark. *Results are the mean ± STD from three replicates of the same plants except for 25% where only one replicate was plotted.*

Figure 5a & 5b shows that the amount of hexose sugars in the dark decreased during drying. In contrast hexose sugar values in the dark for C. wilmsii obtained by the research group of Farrant (unpublished) were much higher especially glucose values (113 ± 24.0 µmol/gDm).

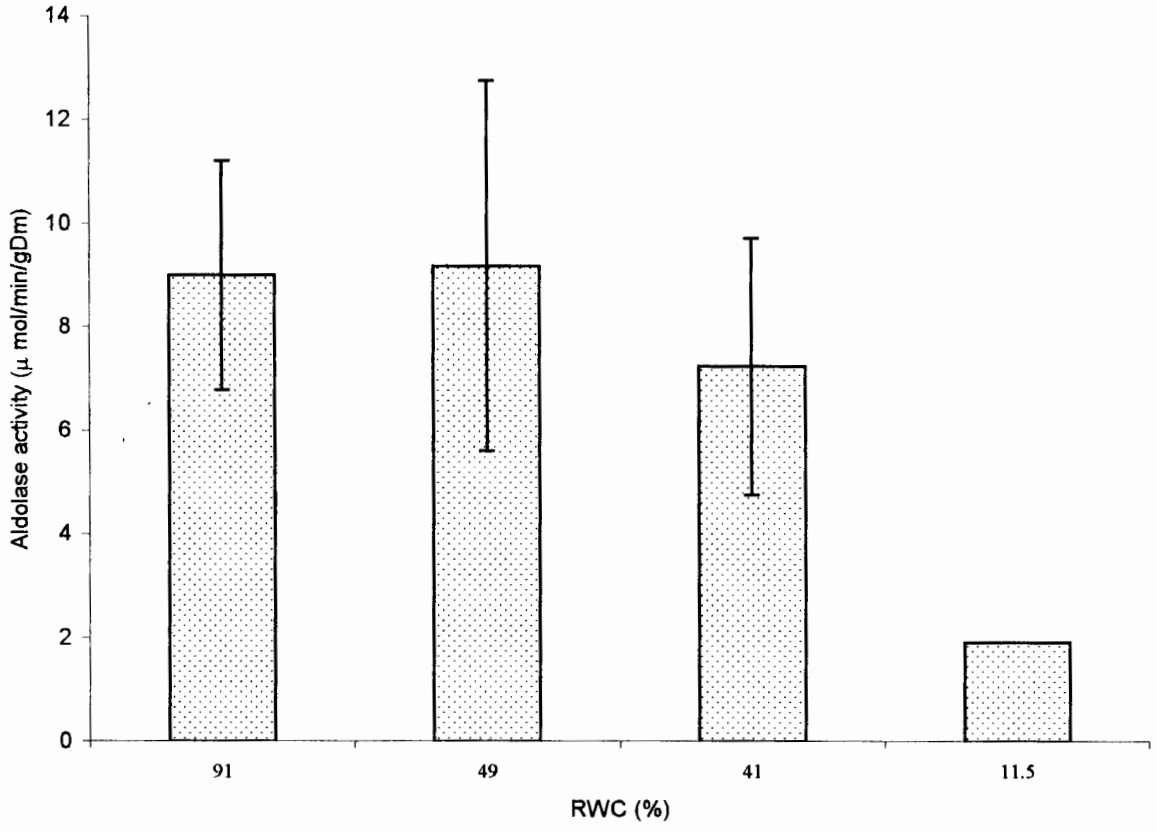

Figure 6: Changes in aldolase activity as RWC decreases for tissues dried in the light. Results are the mean ± STD from three replicates of the same plants except for 11.5% where only one replicate was plotted.

Changes in aldolase activity for plants dried in the light are given in Figure 6. Activity remained constant until 49% RWC after which activity decreased. The lowest activity (1.91 μmol/min/gDm) was recorded for 11.5% RWC which coincide to the point where most metabolic activity start shutting down.

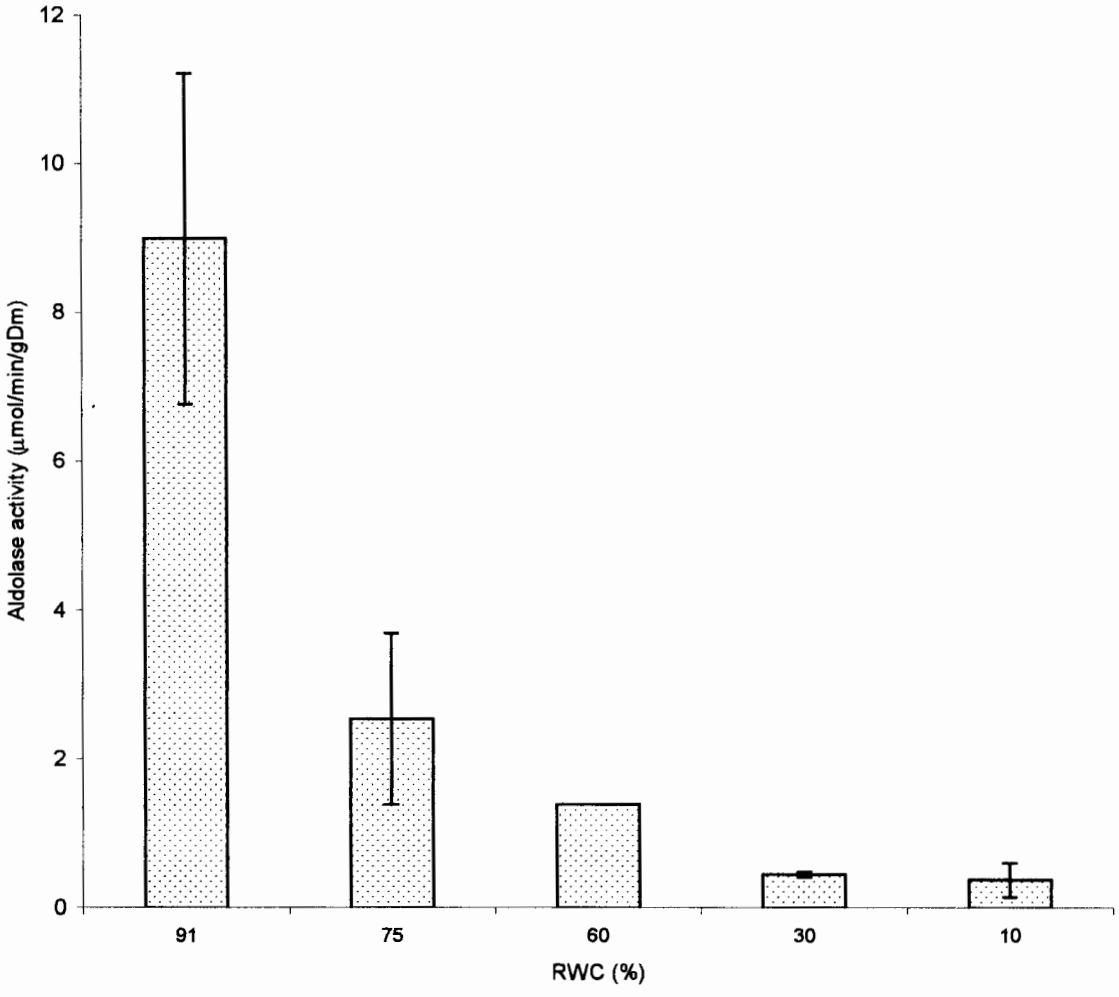

Figure 7: Changes in aldolase activity as RWC decreases for tissues dried in the dark. Results are the mean ± STD from three replicates of the same plants.

Tissues dried in the dark had lower aldolase activity (Fig 7) than for tissues dried in the light. The activity declined through dehydration with only 0.374 ± 0.227 µmol/min/gDm was recorded for 10% RWC as opposed to 2.54 ± 1.15 µmol/min/gDm for 75% RWC.

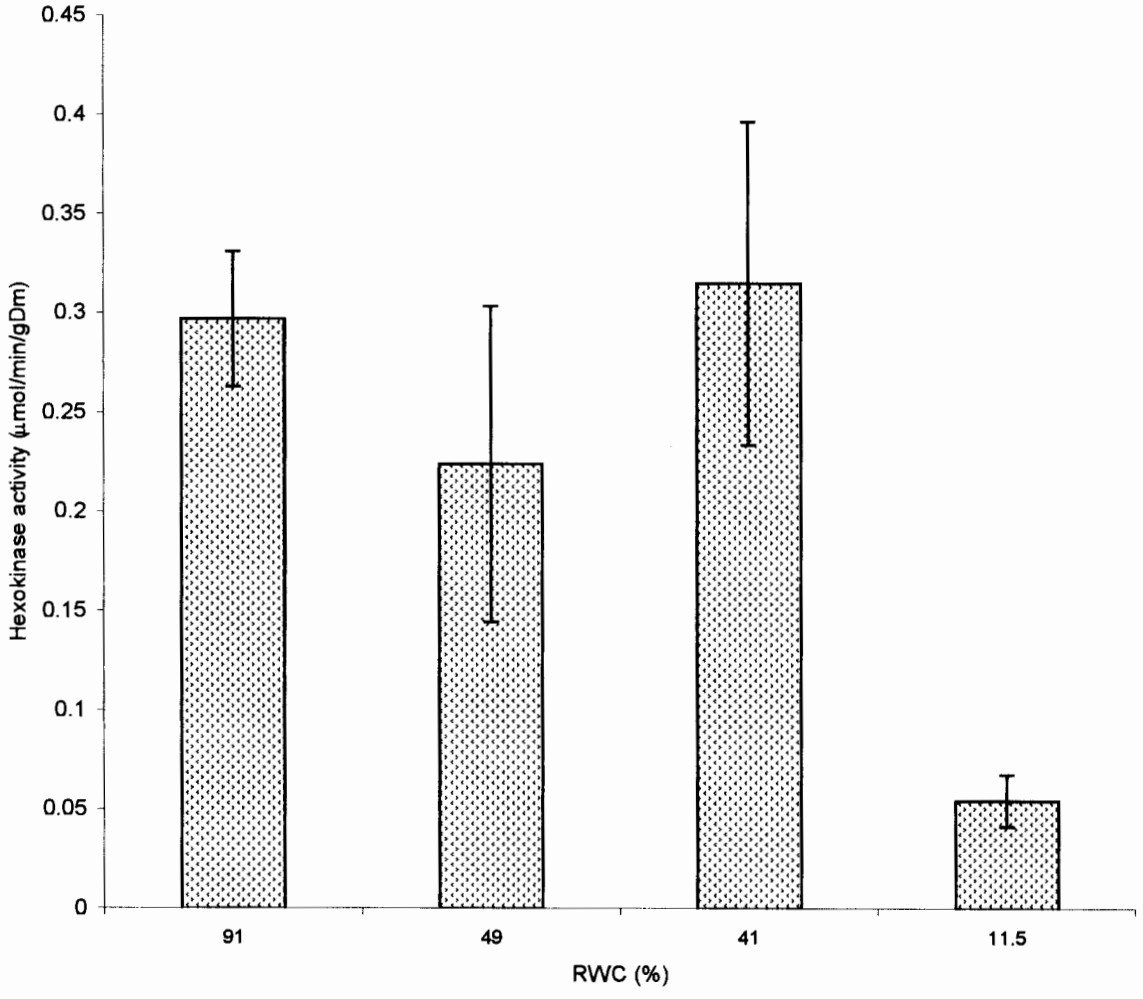

Figure 8: Changes in hexokinase activity as tissues are dried in the light for C. plantagineum. Results are the mean ± STD from three replicates of the same plants.

The data for changes in hexokinase activity of tissues dried in the light are given in Figure 8. There was a decrease in activity from hydrated tissue to 49% RWC followed by an increase at 41% RWC (0.315 ± 0.0814 µmol/min/gDm) and a big drop in activity at lower RWC (0.0544 ± 0.013 µmol/min/gDm).

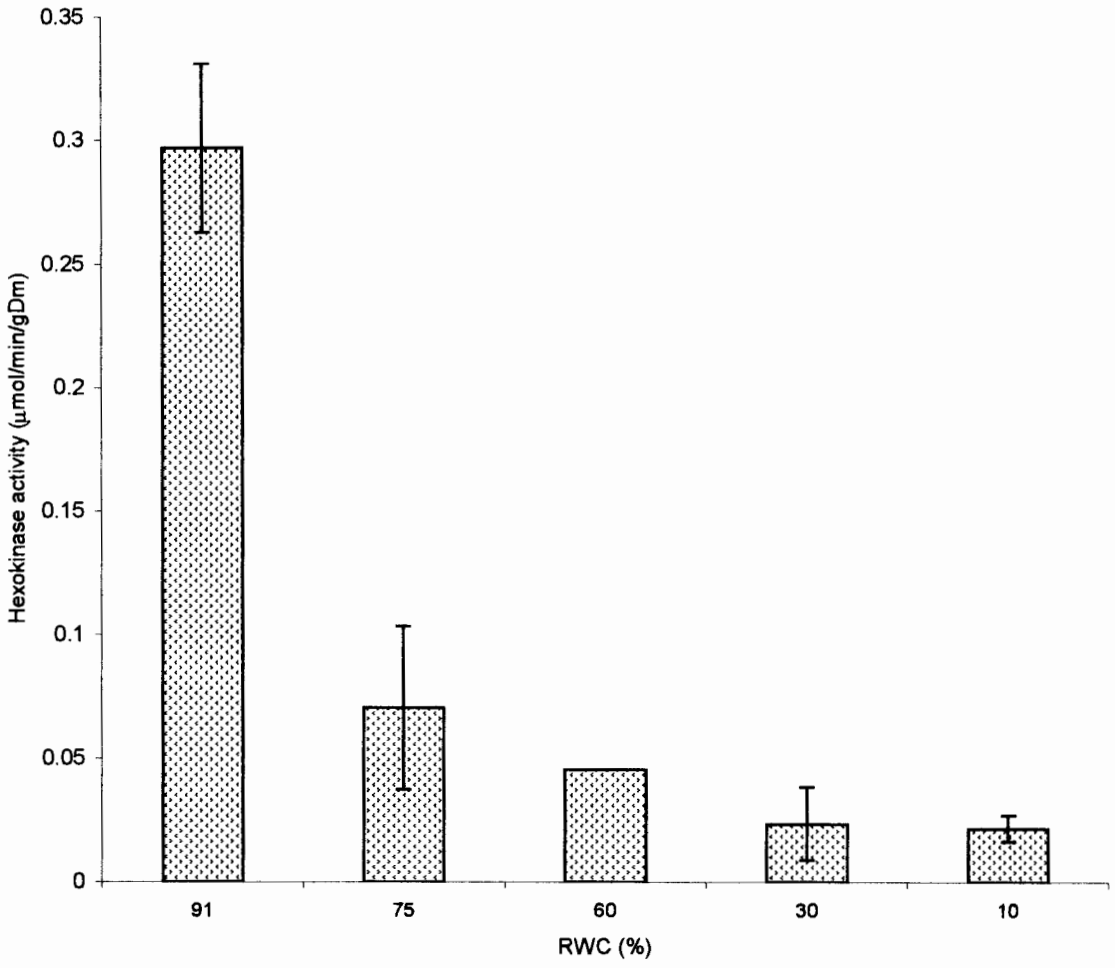

Figure 9: Changes in hexokinase activity as tissues are dried in the dark for C. plantagineum. Results are the mean ± STD from three replicates of the same plants.

The same trend in enzyme activity as for aldolase activity was observed here, for changes in hexokinase activity (Fig. 9). Although, for hexokinase, activity was much lower than aldolase with maximum activity recorded was for the hydrated sample and was only 0.297 ± 0.034 µmol/min/gDm.

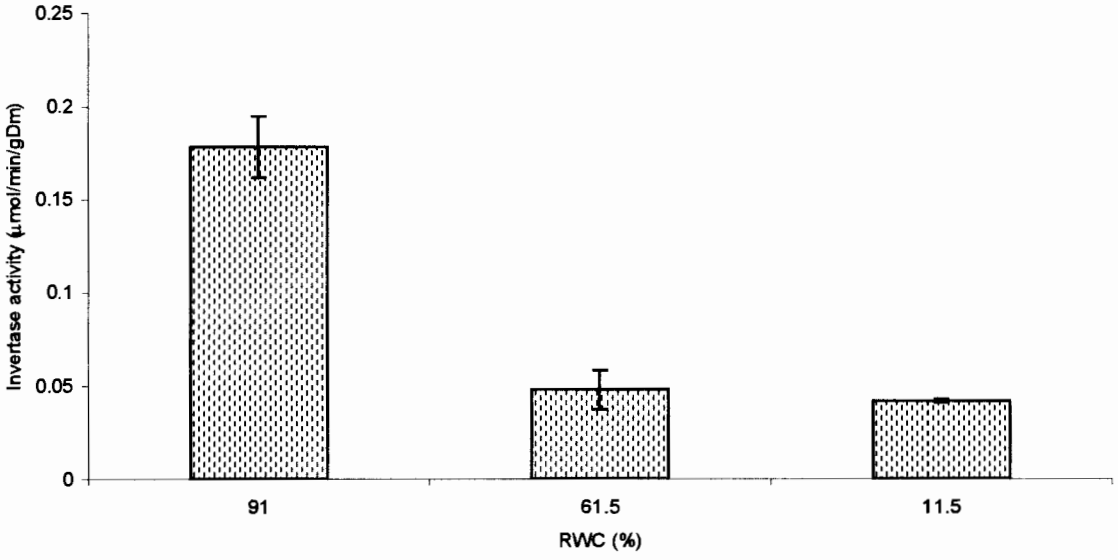

Figure 10: Changes in invertase activity as RWC decreases for tissues dried in the light. Results are the mean ± STD from three replicates of the same plants.

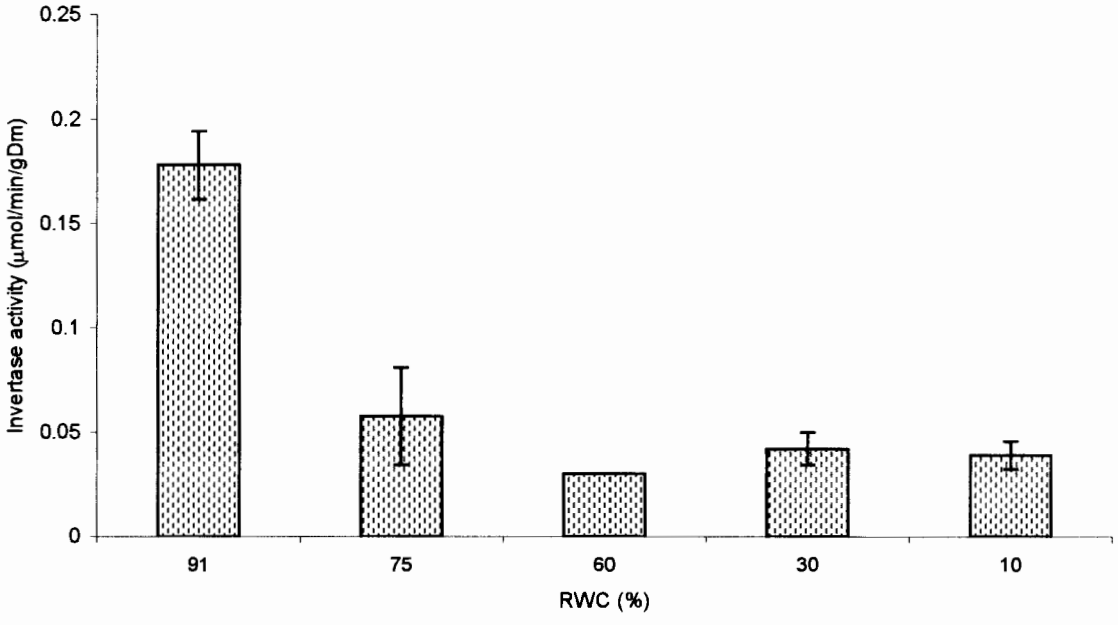

Figure 11: Changes in invertase activity as RWC decreases for tissues dried in the dark. Results are the mean ± STD from three replicates of the same plants

Invertase activity was very low compare to the other two enzymes (Figs 10 & 11). The enzyme was down regulated in both light and dark drying treatments. After drying below 90% RWC the activity level stayed almost the same for both treatments with the lowest value of 0.0419 ± 0.0009 μmol/min/gDm recorded for the light and only 0.0388 ± 0.0068 μmol/min/gDm for the dark.

Discussion

Data obtained for sucrose (Fig. 2), for tissues dried in the light, followed the same pattern observed for *C. wilmsii* (Farrant, unpublished), that is, there was an increase in sucrose accumulation as percentage RWC of the plant decreases. Sucrose content for *C. plantagineum* was significantly higher (689.21 ± 71.42 μmol/gDm at 9.5% RWC) than that observed (Farrant, unpublished) for *C. wilmsii* (445 ± 44.9 μmol/gDm) under the same conditions. Furthermore, sucrose data obtained in this study was also significantly higher than that reported for other resurrection plants such as *X. viscosa*, *X. humilus* and *Sporobulus stapfianus* (Whittaker *et al.*, 2001).

Dehydration in the dark seems to result in a significant decrease in sucrose content (Fig. 4). There was a 9.8-fold decrease in accumulation in the dark. It must be noted that a maximum of 140.34 ± 15.96 μmol/gDm was observed for a RWC of 75%, which seems to be the optimum point for sucrose accumulation in the dark for *C. plantagineum.* The same trend was also observed by research group of Farrant (unpublished) for *C. wilmsii*; though sucrose values obtained for *C. plantagineum* (70.43 ± 9.1μmol/gDm) was much lower than the unpublished sucrose result of Farrant (176 ± 38.0 μmol/gDm) obtained for *C. wilmsii.* This low sucrose value could be one of the possible reasons why resurrection plants dried in the dark lose desiccation tolerance and die.

In contrast to sucrose, hexose sugars data for light dehydrated tissues was significantly lower (Fig 3a & Fig 3b), although a maximum fructose level (231.53 ± 1.25 μmol/gDm) was observed at a RWC of 41%. The low hexose sugar values in dry leaves of *C. plantagianeum*, could account for the high sucrose level obtained under the same conditions. The decrease levels of hexose sugar indicate that glucose and fructose are being phosphorylated to hexose phosphates, which are substrates for sucrose. The same trends in sugar accumulation under light condition were also observed for *C. wilmsii* (Farrant, unpublished). Based on this, it could be hypothesised that the low sugar content in *C. plantagigeum* dried in the dark may have been reflected by an increase in hexose sugar content. However, hexose sugar levels obtained for the dark (Fig 5a & fig 5b) clearly show that glucose and fructose levels remain significantly low, although a higher level of fructose over glucose was observed at 75% RWC. Thus it seems that in the dark negligible amount

of sugars, only 90 µmol/gDm, are accumulated by the plants. It is interesting to note that the maximum sugar content (259 µmol/gDm) in the dark was obtained at 75% RWC. In contrast, unpublished data obtained by Farrant, for C. wilmsii, indicate a higher glucose level (113 ± 240 µmol/gDm) as opposed to fructose (27.3 ± 8.5 µmol/gDm) for the dark. This suggests that not all glucose and fructose molecules were incorporated into sucrose thus accounting for the low sucrose level (176 ± 38.0 µmol/gDm) obtained for *C. wilmsii*. However, the same conclusion cannot be drawn for *C. plantagineum* since the hexose sugar values for the dark remain very low compared to *C. wilmsii* under the same conditions. The reasons for this differences in the trends of hexose sugar content in the dark between *C. plantagineum* and *C. wilmsii* needs further investigation, It would appear that in *C. plantagineum*, other carbon sources beside glucose and fructose may be contributing to the very high sucrose values obtained in the light.

The resurrection plant *C. plantagineum*, accumulates (beside sucrose) large amount of an 8-carbon sugar, 2-octulose (Oliver *et al.*, 1998,Norwood *et al.*, 1999; Norwood *et al.*, 2000, Scott, 2000). According to Norwood et al (2000), the 2-octulose produced is used as a soluble storage carbohydrate in the leaves, which is mobilised at night. Furthermore, it has also been suggested that 2-octulose is a major carbon source for the production of sucrose during dehydration in *C. plantagineum* (Norwood *et al.*, 1999). 2-octulose was also reported for *C. wilmsii* by Norwood et al (2000), however data was not shown. The metabolism of 2-octulose to sucrose involves a range of enzymes associated with the gluconeogenesis pathway. Aldolase is one of the enzymes in this pathway. Aldolase is suggested to metabolise octulose 1,8-bisphosphate, which is an intermediate in the conversion process (Norwood *et al.*, 2000).

Aldolase activity levels in tissues dried in the light (Fig 6) were consistent although a drop in activity level was recorded at lower RWC (11.5%). The aldolase activity observed in the light (hydrated sample) was comparable to the data obtained by Norwood et al (1999) for hydrated samples. However further comparison could not be made since Nowwood *et al* (1999), only performed a partial dehydration on *C. plantagigeum* as opposed to complete dehydration used in this study. Moreover Norwood *et al.* (2000) showed diurnal regulation of aldolase activity over a 24h period on *C. plantagineum* with an increase in aldolase activity over the light period. However, since plant samples were harvested in the morning

in this study it seems more likely that the trends observed (Fig. 6) were the effect of dehydration rather than diurnal rhythm for the light. Furthermore, Norwood *et al* (2000), also pointed out that 2-octulose is also a product of photosynthesis. Consequently, since an increase in sucrose accumulation (Fig 2) was obtained in the light, it could be possible that octulose produced during the early stages of dehydration (light) by photosynthesis, contributed to the huge sucrose content observed.

In contrast, aldolase activity level for dark dehydrated tissues (Fig. 7) was substantially down regulated with a 24-fold decrease in activity. It must be noted that this has not been reported before. Furthermore, this data coincides with a significant drop in sucrose accumulation (Fig.4) under the same conditions. As reported previously, this enzyme has a diurnal rhythm with a high activity level during the day followed by a fall in activity level at the start of the night and rise again later through out the night (Norwood *et al.*, 2000). Therefore, it would appear that the substantial decrease in aldolase activity would very likely affect octulose conversion to sucrose in the dark. Moreover, since octulose was not measured in this study, the extent to which a decrease in aldolase activity affects sucrose metabolism cannot be established. This will require further investigation. Moreover, the reason why aldolase activity decreases in the dark is not fully understood, it might be that the enzyme is affected by this diurnal rhythm since the dehydration process for this study was carried out over a two weeks period in the dark. The only evidence reported so far is that octulose metabolism and related enzyme activity appear to be regulated by light (Norwood *et al.*, 2000)

Hexokinase is the enzyme responsible for the conversion of glucose and fructose to hexose monophosphate, which is the substrate responsible for sucrose synthesis. In the present study, hexokinase activity level for tissues dried in the light was more or less maintained at a constant level between 91% to 41% RWC (Fig 8). This coincides with a decrease in glucose and fructose level although an increase in fructose content was observed at 41%RWC (Fig.3b). This does not change the fact that the level of hexokinase was probably sufficient to allow the conversion hexose sugar to sucrose in the dry state. Similar results were observed on other resurrection plants, *X. viscosa* and *Sporobulus stapfianus* (Whittaker *et al.*, 20001). However, hexokinase activity level was much higher in these two resurrection plants than what was observed for *C. plantagineum* dried in the light for this study. Furthermore, work done by Norwood *et al.* (2000), also showed that compared to

aldolase, hexokinase seems to be unaffected by day and night rhythm since hexokinase activity remains constant through out the experiment.

However, in the dark hexokinase activity was significantly reduced (Fig 9). Since no build up hexose sugars was observed (Fig 5a & Fig 5b), it seems that hexokinase does not play a major role in the decrease of sucrose observed (Fig 4) in the dark. Compared to *C. plantagineum*, unpublished data obtained for *C. wilmsii* by Farrant showed a build up of hexose sugars and a decrease in sucrose accumulation (176 ± 38.0 μmol/gDm) in the dark. Whether a decrease in hexokinase activity is responsible for the significant build up of hexose sugars in the dark dehydrated tissue of *C. wilmsii* remains to be investigated. Thus, it does appear that *C. plantagineum* does not rely solely on phosphorylation of glucose and fructose for its sucrose. Therefore, it seems more likely the plant depends on other carbon sources for obtaining its huge sucrose content for example 2-octulose.

Acid invertase activity was also measured in the project, in an attempt to explain the differences obtained in sucrose accumulation between the light and dark sample. The function of acid invertase enzyme is to hydrolyse sucrose to glucose and fructose. Consequently, failure to down regulate acid invertase in the dark would contribute to lower sucrose content. Alternatively, a decrease in invertase activity would result in higher sucrose accumulation in both light and dark. Low invertase activity was observed for light tissue dehydrated in the light. This could be a contributing factor to the high sucrose content obtained in the light (Fig 2). However, in the dark sucrose content remains very low despite the low invertase activity observed. Consequently, the major differences in sucrose content between light and dark dehydrated samples cannot be due to differences in measurable invertase since invertase data for both light and dark (Fig 10 & Fig 11) are comparable.

Therefore, it seems that the low sucrose value observed in dark dehydrated samples of *C. plantagineum* is due to the low level of activity recorded for aldolase in dark dehydrated samples. What is causing a decrease in aldolase activity? Further investigation is required to be able to answer this question. Furthermore, other enzymes associated with the

gluconeogenesis pathway should also be measured so as to get a better understanding of the pathway itself and also on the metabolic effects of the plant when dehydration is carried out in the dark.

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